December 2, 1998

PROPOSED VICH PROCEDURE FOR THE DETECTION OF MYCOPLASMA CONTAMINATION

General procedure for detecting mycoplasma contamination

Each batch of live viral vaccine, each lot of master seed virus (MSV), each lot of primary and master cell stock (MCS), and all ingredients of animal origin not steam sterilized or irradiated should be tested for the absence of mycoplasmas. Solid and liquid media such as Hayflick's, Frey's, or the 113.28 Heart Infusion mycoplasma media, as well as an indicator cell line, such as African green monkey kidney (VERO) cells, should be used to detect mycoplasma contamination. These media's and cell lines should be able to detect small numbers of test organisms including *Acholeplasma laidlawii* (ATCC # 23206), *Mycoplasma arginini* (ATCC #23838), *M. hyorhinis* (ATCC # 17981), and *M. orale* (ATCC # 23714). For avian biologicals, the test organism *M. synoviae* (ATCC # 25204) should also be used. The nutritive properties of each lot of solid medium should have CFUs within one standard deviation of the established count for each frozen or lyophilized lot of the above listed test organisms. An appropriate color change should occur in the liquid media when approximately 20 CFUs of each test organism are inoculate. Stained micro colonies of mycoplasma should be visible on the indicator cell at the endpoint dilution's established for each of the above listed test organisms.

One sample of each batch of vaccine should be tested. Inoculate one agar plate for each final batch of vaccine with 0.1 ml of the sample being tested and inoculate 100 ml of the selected liquid medium with 2 ml of the sample. Incubate the plates from final batches of vaccine at 35-37°C aerobically (an atmosphere of air containing 4-6 % CO_2 and adequate humidity) for 10-14 days. On day 7 and 14 after inoculation, subculture 0.1 ml from the liquid media onto 1 agar plate each. Incubate each plate aerobically for 10-14 days. If MSV, MCS, or ingredient of animal origin is being tested, inoculate 2 agar plates each of at least 2 selected media with 0.1 ml of the sample being tested. Incubate all plates at 35-37°C, and for each media incubate one aerobically and the second anaerobically (an atmosphere of nitrogen containing 5-10 % CO_2 and adequate humidity) for 10-14 days. Inoculate 2 ml of each sample into 100 ml of each of the selected broth media. Inoculate 0.05 ml of the sample into 2 wells of chambered slides (example; Lab-Tek) containing the indicator VERO cells and incubate aerobically for 3-5 days. On day 7 and 14 after inoculation, subculture 0.1 ml from each of the 2 liquid media onto 2 agar plates of the 2 selected media. Incubate 1 plate of each selected media aerobically and 1 plate anaerobically for 10-14 days. Incubate the inoculated liquid media at 35-37°C and observe periodically throughout the 14 days of incubation and if any color change occurs, subculture immediately.

Interpretation of mycoplasma test results

At the end of each 10-14 day incubation period examine all the inoculated solid media microscopically for the presence of mycoplasma colonies. The test sample passes the test if the growth of mycoplasma colonies has not occurred on any of the inoculated media. If at any stage of the test, more than one plate is accidentally contaminated with bacteria or fungi, or is broken, the test is invalid and needs to be repeated. If mycoplasma colonies are found on any agar plate, the test should be repeated once to confirm the mycoplasma contamination. If mycoplasma colonies are found on any of the agar plates of the retest, the test sample should be considered unsatisfactory because of mycoplasma contamination. After 3-5 days of incubation the VERO cell indicator chamber slides should be stained with a DNA fluorochrome stain (example; Hoechst Bisbenzamid), and if any of the sample chambers have micro colonies when examined microscopically this is a positive presumptive test for mycoplasma contamination, which must be confirmed. If the agar(s) used with this presumptive positive test does not show mycoplasma colonies the test needs to be repeated using additional formulations of mycoplasma media, and PCR in order to confirm mycoplasma contamination that was non cultivable on the first agar(s) used.

Points for discussion:

1. Are two incubation conditions (aerobic and anaerobic) necessary when testing final batches of vaccine?
2. Which media are acceptable for final product, MSV, MCS, and ingredient testing; Hayflick's, Frey's, Frii's, 9CFR 113.28, ATCC, M-96, etc.?
3. Should there be 1 standard media or a choice of 2 or more media?
4. Should exact formulations for media be specified in the standard method, or is it sufficient to require the growth of the test organisms?
5. Does each lab need to initially compare the growth promotion of their test organisms against an international set of titered test organisms? If yes then how often, yearly?
6. Should the testing of new lots of raw ingredients for growth promotion be required?
7. Which media are acceptable for poultry; vaccine, MSV, and MCS testing?
8. Does DPN-cysteine need to be required in media used for poultry vaccine testing?
9. Should T-strain testing be required on final batches, MSV, MCS, and/or ingredients of animal origin?
10. Should the broth inoculum be 1 ml, 2 ml or 10 ml?
11. Should the inoculum onto the agar be 0.1, 0.2 or 0.25 ml?
12. Would there be antibiotic (Gentamicin) inhibition of mycoplasma growth with the larger 10 ml inoculum?
13. Should PCR be used as a screening, confirmatory or final test?
14. Should there be different testing requirements for MSV, MCS, and final batches?
15. Should there be a list of media and production ingredients which need to tested for mycoplasma contamination?
16. Should the standard method call for repetitive looking at the same plates for 28 days or multiple plates for each subculture which can be looked at and thrown over the course of the 28 days of the test?
17. Should the agar plates be examined throughout the 28 days (at 3, 7, 10, 14, 21 days) or just on the 28th day of the test?
18. Should there be a requirement to test killed viral products for mycoplasma contamination before they are inactivated?
19. Should the indicator cell/DNA stain procedure be included as a standard procedure for non cultivable mycoplasma detection? What tests should be used to confirm noncultivable mycoplasma?
20. How will this mycoplasma standard setting VICH committee judge whether the adding of a new test or additional media is cost effective?

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VICH DISCUSSION DOCUMENT

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VICH Biological Quality Monitoring WG
Topic: Mycoplasma Testing

COMMENTS ON THE PROPOSED POINTS FOR DISCUSSION

1. Are two incubation conditions (aerobic and anaerobic) necessary when testing final batches of vaccine? Both are necessary, but change anaerobic to microaerophilic. Both. Only aerobic. Both. Both but presently use only aerobic.

2. Which media are acceptable for final product, MSV, MCS, and ingredient testing; Hayflick's, Frey's, Frii's, 9 CFR 113.28, ATCC, M-96, etc.? Would like the option of omitting the preservatives (penicillin and thallium acetate) to eliminate inhibitory activity on certain mycoplasmas. Option of adding different species serum and NAD when required.

3. Should there be 1 standard media or a choice of 2 or more? It depends on the set of control organisms, if one media can grow all the control organism then go with 1. At least 2. One standard with additional for MCS, MSV, and ingredients. One if it can be validated with control organisms. Choice of 2 or more media. One media selected on the growth of common mycoplasma in the specific country.

4. Should the exact formulations for media be specified in the standard method, or is it sufficient to require the growth of the test organisms? Additives need to be described since many of the additives are derived from live materials and their properties may be different in each country. Exact formulations are not necessary if growth of indicator organisms is required. It would be useful to have exact formulations as a reference, but deviations of some additives such as serum and NAD should be allowed if the control organisms grow at the required level.

5. Does each lab need to initially compare the growth promotion of their test organisms against an international set of titered test organisms? If yes, then how often, yearly? This would be the preferred situation but how can it be accomplished? Ideal but difficult to establish and control. Specify ATCC numbers and limit passage and CFUs. Yes this is preferred. One institution would have to batch distribute the strains and then each institution would have to confirm that they equally proliferate in a specified medium, a yearly check would not be necessary since the proliferation rate can be confirmed by establishing a positive control at the time of the certification test.

6. Should the testing of new lots of raw ingredients for growth promotion be required? The proliferation rate should be confirmed when new media, additives or serum is used. Yes. No, as long as each lot of media is tested by the accepted indicator organisms. Not necessary if final media formulation meets the acceptable standard required using the standard control organisms.

7. Which media are acceptable for poultry; vaccine, MSV, and MCS testing? Any media as long as it contains NAD (DPN-cysteine). Frey media is the most acceptable. A media where MG and MS can proliferate.

8. Does DPN-cysteine need to be required in media used in poultry vaccine testing? Yes. Yes plus swine serum. Recommend DPN-cysteine, especially for *M. synoviae.*

9. Should T-strain testing be required on final batches, MSV, MCS, and/or ingredients of animal origin? This would require another media formulation but could be carried out on MCS and MSV. Yes, *Ureaplasma gallorale* was described in Asia. Testing only on MCS and MSV. Why was *Ureaplasmas* removed from the EP indicator organism list? The necessity of testing deserves discussion.

10. Should the broth inoculum be 1, 2, or 10 ml? Adequate to inoculate at 1-2% of medium since there is an anti microbial influence with a large inoculum. 1 ml. 10 ml. 2 ml in 100 ml of medium is the maximum without antibiotic interference. 10 ml. 2 ml, it depends on the level of sensitivity you are trying to achieve.

11. Should the inoculum onto agar be 0.1 ml, 0.2 ml, or 0.25 ml? 0.1 ml. 0.1 ml on a 15X60 mm takes 1-2 hrs to dry, whereas 0.2 ml takes all day to dry before the plates can be inverted and placed in the incubator. 0.25 ml. 0.1 ml, larger amounts leads to problems of contamination because of capillary action around the edges of the plates.

12. Would there be antibiotic (Gentamicin) inhibition of mycoplasma growth with the larger 10 ml inoculum? Whatever the volume of inoculum, in the presence of antibiotics there can be inhibition of mycoplasma, so recommend dilution or neutralization. With inoculum larger than 2 ml there is inhibition of *M. orale*, if the 10 ml inoculum were adopted, a section would need to be added to validate the volume of media needed. It's adequate to inoculate at 1-2% of the medium.

13. Should PCR be used as a screening, confirmatory or final test? The PCR method will be the new standard procedure done in parallel to the culture method, a confirmation test. PCR is not necessary to be done, because it is sufficient to detect only live mycoplasma. Use PCR as another alternative test to the indicator cell method. PCR is good but has limitations because of additional personnel time and need for additional separate isolation space. PCR could be used as a complementary test for MCS, MSV, and animal ingredients and as a screening test for final batches, if validated. Literature indicates that 22/61 commercial, live veterinary vaccines were positive by PCR, although no viable mycoplasma cells could be isolated.

14. Should there be different testing requirements for MSV, MCS, and final batches? Not required. Same testing requirements for all samples. Additional media and methods are needed for MCS and MSV. Need to have a test for non-cultivable mycoplasma but need guidelines for a positive result. Yes. Need provision for the addition of critical additives for testing MCS and MSV. Use the indicator cell/DNA stain procedure as an early warning method of mycoplasma contamination.

15. Should there be a list of media and production ingredients which need to be tested for mycoplasma? Test all ingredients of animal origin except those steam sterilized or irradiated. Yes. No list needed. Not required. No not necessary.

16. Should the standard method call for repetitive looking at the same plates for 28 days or multiple plates for each subculture which can be looked at and thrown away over the course of the 28 days of the test? Repetitive looking preferred over multiple plates. Repetitive looking for 21 days.

17. Should the agar plates be examined throughout the 28 days (3, 7, 10, 14, 21 days) or just on the 28th day? Read each set on a 10 day minimum. Yes examine throughout the 21 days. Examine the plates throughout 28 days. Observe at 14 days only. Examine on 3-4 day intervals before the incubation is finished at 10-14 days. Specify the time to the end of the subculture as the test period.

18. Should there be a requirement to test killed viral products for mycoplasma contamination before they are inactivated? This test is applied to live vaccines not inactivated. No, its not necessary because MSV, MCS, and ingredients are examined for mycoplasma contamination. Yes. No. Yes. It would be reasonable to test killed product before inactivation.

19. Should the indicator cell/DNA stain procedure be included as a standard procedure for non-cultivable mycoplasma detection? What test should be used to confirm non-cultivable mycoplasma? We have detected non-cultivable and cultivable by indicator cell procedures, serves as early warning, recommend to include. Recommend PCR when it exists. If incidence of non-cultivable is large enough then the use of the test is warranted. Suggest using PCR to confirm positive DNA stain. The DNA stain is not required since the PCR test has been proposed.

20. How will this mycoplasma standard setting VICH committee judge whether the adding of a new test or additional media is cost effective? Cost effective method should be selected by VICH. It should be considered whether the adding of a new test or additional media are cost effective. Concerned that the committee will not even consider cost effectiveness. Reply will come from available data and a discussion with industry which certainly has information or experience about the problem. Primary concern is from a scientific perspective, substantiating data required.

VICH DISCUSSION DOCUMENT

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VICH Biological Quality Monitoring WG
Topic: Mycoplasma Testing

COMMENTS ON THE PROPOSED TEST PROCEDURE

1. Two commented that the format of the procedure was confusing and felt that a procedure should be put in a step wise format rather than paragraphs.

2. A comment suggested that the 4 organisms be used to validate the assay and then use only one organism as a positive control.

3. A comment suggested to add a subculture step to the VERO cell procedure to increase the possibility of detection.

4. One comment suggested 100 CFUs and another suggested 20 - 40 CFUs instead of the procedures suggested 20 CFUs for the indicator organisms challenge concentration.

5. One comment suggested the inoculation of 2 plates initially and at each subculture so that if one is broken or bacterially contaminated, another plate would be available for observation.

6. One comment thought it was confusing to use 1 media and 1 incubation condition for final product and 2 media and 2 incubation conditions for MCS and MSV.

7. One comment felt that a subculture should be done at 3 days to better isolate the rapid growers.

8. One comment was concerned with the criteria for a valid test. How many of the indicator organisms have to cause a color change in the broth with 20 CFUs? What's a valid VERO cell indicator test? What media would be used to establish the counts for each indicator organism?

9. One comment wondered what the criteria would be for the 2nd media to be used to test MCS, MSV or ingredients?

10. One comment asked if a sample would be rejected as unsatisfactory if it was positive by the indicator cell/DNA stain but were not able to demonstrate colonies?

11. One commentor asked that the procedure be more specific in the appropriate use of positive and negative controls in each aspect of the test.

12. One commentor indicated that a color change in the broth is not always seen and sometimes turbidity is of importance.

13. One commentor preferred to start with a comparison of the different existing tests instead of a proposed procedure.

14. One commentor felt the inoculation volumes were not high enough (10 ml and 0.25 ml) and the observation period was not long enough.

15. One commentor preferred to have the procedure be a negative presumptive test rather a positive for mycoplasma contamination procedure.

16. One commentor felt that the possibility of mycoplasma contamination during processing was small and a test on each process step shouldn't be required. Most of the mycoplasma contamination is from preparation materials.

17. One commentor felt that the mycoplasma control organism should be mycoplasma pathogenic to each animal.

18. One commentor felt that each country should stipulate what process steps should be subjected to mycoplasma testing.

19. One commentor felt that it should be clarified as to what medium and positive control strain of mycoplasma should be used for testing vaccines for each animal species.

Example; *A. laidlawii* for testing when an anti microbial is present, *M. hyorhinis* for vaccines other than poultry, *M. gallisepticum, M. pneumoniae* and other strains fermenting D-glucose for poultry vaccine, etc.

20. One comment suggested that the procedure express the specifics for a valid test and well as what was a invalid test.

21. One commentor specified that the new proposed PCR test in Japan combines PCR with a proliferating culture method, which is to be used in parallel with the direct culture method.

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TABLE 1: Comparison of Current Regulations

PROCEDURE/SPEC	JAPAN	EU/CVMP	USA
Procedure	Minimum Requirements of Biological Products for Animal Use	European Pharmacopoeia Supplement 1998, 2.6.7	9 CFR 113.28 SAM 910
Broth inoculum	1 ml (1 dose?)/100 ml	10 ml/100 ml	1 ml/100 ml
Agar inoculum	0.1 ml	0.2 ml	0.1 ml
Number of vials tested	2 or more vials	$\geq$4 but $\leq$10 vials	1 vial
Days of incubation	14 days/broth 10 days/plate 24 days total incubation	21 days/broth 21 days/plate 35 days total incubation	14 days/broth 10-14 days/plate 28 days total incubation
Number of agar plates and days of inoculation	0, 3, 7, 10, 14 days 1 plate/day	1, 2, or 3 days/2 plates 6, 7, or 8 days/2 plates 13 or 14 days/2plates	0, 3, 7, 10, 14 days 1 plate/day
Incubation condition(s)	5% CO_2 in air	Aerobic: 5-10% CO_2 in air Microaerophilic: 5-10% CO_2 in nitrogen	4-6% CO_2 in air
Incubation temperature	35-37°C	35-38°C	33-37°C
Media	Bovine myocardial infusion	Beef heart infusion or PPLO	Heart infusion
Serum in media	Equine and porcine	Equine and porcine	Equine
Preservatives	Penicillin G potassium and potassium acetate	Penicillin and thallium acetate	Penicillin and thallium acetate
Color indicator	Phenol red	Phenol red	Tetrazolium chloride
Positive controls	*M. gallisepticum* *M. synoviae* *M. hyopneumoniae* *M. orale* (100 CFU, broth & agar)	*A. laidlawii* *M. gallisepticum* *M. hyorhinis* *M. orale* *M. snyoviae* (100 CFU/plate, 40 CFU/broth)	Selected mycoplasma cultures (number of organisms not specified)
Judgement	No colonies of mycoplasma from product, but colonies of *M. snyoviae* on control	No mycoplasma in any product inoculated media	No mycoplasma colonies on any plate inoculated with product

PROCEDURE/SPEC	JAPAN	EU/CVMP	USA
Repeat test	No colonies of *M. synoviae*, but colonies from product	If broth has bacteria or fungi contamination If at any stage both plates are contaminated or broken If growth of mycoplasma from product, test may be repeated once using twice the inoculum, media and plates	No growth on positive controls or growth on negative controls
Indicator cell culture method	No	Yes	No
PCR method	Proposed	No	No
Tests for MSV, MCS, working cells, or control cells	Culture method	Both culture method and indicator cell culture method	Culture method
Tests for virus harvest, bulk, final product	Culture method	Culture method	Culture method
Tests for inhibitory substances	No method specified	Positive control organisms in presence of product	No method specified